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## Bioenergetic Consequences of Lactose Starvation for Continuously Cultured *Streptococcus cremoris*

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*Streptococcus cremoris* cells that had been grown in a chemostat were starved for lactose. The viability of the culture remained essentially constant in the first hours of starvation and subsequently declined logarithmically. The viability pattern during starvation varied with the previously imposed growth rates. The death rates were 0.029, 0.076, and 0.298 h<sup>-1</sup> for cells grown at dilution rates of 0.07, 0.11 and 0.38 h<sup>-1</sup>, respectively. The proton motive force and the pools of energy-rich phosphorylated intermediates in cells grown at a dilution rate of 0.10 h<sup>-1</sup> fell to zero within 2 h of starvation. The culture, however, remained fully viable for at least 20 h, indicating that these energy-rich intermediates are not crucial for survival during long-term lactose starvation. Upon starvation, the intracellular pools of several amino acids depleted with the proton motive force, while large concentration gradients of the amino acids alanine, glycine, aspartate, and glutamate were retained for several hours. A quantitative analysis of the amino acids released indicated that nonspecific protein degradation was not a major cause of the loss in viability. The response of the energy metabolism of starved *S. cremoris* cells upon refeeding with lactose was monitored. Upon lactose starvation, the glycolytic activity and the rate of proton motive force generation decreased rapidly but the steady-state level of the proton motive force decreased significantly only after several hours. The decreasing steady-state level of the proton motive force and consequently the capacity to accumulate amino acids after the addition of lactose correlated well with the loss of viability. It is concluded that a regulatory loss of glycolytic capacity has a pivotal role in the survival of *S. cremoris* under the conditions used.

In their natural habitats, microorganisms are to various extents exposed to fluctuations in the availability of substrates. Some organisms are better adapted to conditions of nutrient starvation than others. The oligotrophic and copiotrophic marine bacteria are examples of organisms with extreme longevity (1, 25). On the contrary, the facultative and obligate anaerobic chemoheterotrophs studied thus far, i.e., *Streptococcus lactis* (36), *S. faecalis*, and other *Streptococcus* species (31), *Staphylococcus epidermidis* (11), *Peptococcus prevotii* (30), *Ruminococcus flavefaciens* (45), and *Selenomonas ruminantium* (22, 23) are more sensitive to nutrient starvation and lose their viability within a few days.

Factors that have been implicated in the survival of microorganisms during nutrient starvation are the possession of storage compounds (5), the presence of substrates for endogenous metabolism (4), the growth-limiting nutrient before starvation (22, 23, 29), the adenylate energy charge (12, 14), the energy of maintenance requirement (5), the maintenance of a proton motive force (16), and diminution of cell size (13, 25), and various other environmental and organism-specific parameters.

The cytoplasmic membrane is the major barrier for separating the interior of the organism from its environment. To maintain this osmotic barrier function, which is vital for various cellular processes, the cell requires metabolic energy. Several sources of metabolic energy can be involved in the transport of solutes across the cytoplasmic membrane. The driving force for most solutes is the proton motive force or one of its components. Some solutes are translocated by ATP-dependent transport systems, whereas the uptake of some sugars can be mediated by phosphoenolpyruvate (PEP)-dependent group translocation systems (15).

In this study, *S. cremoris* was used to investigate the effect of lactose starvation on several energetic and membrane-associated functions. *S. cremoris* is a strictly fermentative lactic acid bacterium which can ferment only a few carbohydrates. Substrate-level phosphorylation yields ATP, which is used partly to generate the transmembrane electrochemical proton gradient. *S. cremoris* is unable to consume endogenous substrates or to invoke any specialized mechanism to survive periods of nutrient starvation. Survival of energy starvation by *S. lactis* has been attributed to the maintenance of a large PEP pool (5, 43). These organisms do not possess metabolic energy in other storage compounds, which allows an exact determination of the transition from growth to energy starvation. This paper shows that continuously cultured (lactose-limited) *S. cremoris* cells are rapidly depleted of all energy-rich intermediates upon lactose starvation. The maintenance of large concentration gradients for a number of amino acids in the absence of metabolic energy appears to depend on the corresponding transport mechanism. Culture viability and secondary transport activity in starved cells correlate with the steady-state level of the regenerated proton motive force. The capacity to generate a proton motive force decreases during starvation owing to loss of glycolytic activity.

### MATERIALS AND METHODS

**Organisms and culture conditions.** *S. cremoris* Wg2 was obtained from the Netherlands Institute of Dairy Research, Ede, The Netherlands. Strains were grown in batch culture by transferring the organisms from milk cultures to a complex MRS medium (6) containing 1% (wt/vol) lactose. Growth proceeded overnight at 30°C. For the continuous-culture experiments, about 20 ml of an overnight culture of *S. cremoris* Wg2 was transferred to chemically defined medium (RFP) as described by Otto et al. (27). The medium

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contained 0.5% (wt/vol) lactose as the energy source unless stated otherwise. The pH of the medium was controlled at 6.4 by titration with 1 or 2 N NaOH. Chemostat cultures were grown anaerobically under a  $N_2$  atmosphere in glass fermentors, each containing a working volume of 250 ml at 30°C. The culture vessel was equipped with a side tube sealed with butyl rubber which could be pierced by a syringe needle for rapid sampling, i.e., quenching of the metabolism.

**Preparation of bacterial suspensions for starvation experiments.** Lactose starvation was carried out either in growth medium or in a phosphate-lactate buffer containing 50 mM potassium phosphate (pH 6.4), 1 mM  $MgSO_4$ , and sodium L-lactate in a concentration equal to that of the growth environment. The lactate concentration was usually around 45 mM in the steady state of a lactose (0.5%)-limited chemostat of *S. cremoris* Wg2. Lactose starvation in culture fluid was imposed by switching off the medium supply to the continuous culture. For starvation in the phosphate-lactate buffer, cells were harvested by centrifugation ( $24,000 \times g$ ; 1 min), washed twice with the buffer, and finally resuspended to approximately 0.3 mg of protein per ml. Subsequently, the cells were transferred to sterile screw-cap flasks and incubated at 30°C, unless stated otherwise. The whole procedure took about 30 min and was performed aseptically. Essentially the same procedure was used for starvation of batch-grown cells.

To monitor the response of starved cells to lactose, samples of 25 ml were withdrawn from the culture, washed when indicated, and transferred to 30-ml flasks. The cell suspensions were incubated on a rotary shaker at 30°C. Lactose (0.1% wt/vol) was added at the times indicated in the text or legends to figures.

**Viability measurements.** (i) **Plate count method.** The culture suspensions were diluted stepwise with either the effluent of the chemostat from which the cells had been removed or the phosphate-lactate buffer as described above. Finally, 50 to 100  $\mu$ l of cell suspension was spread on agar plates containing MRS medium, 1.5% agar (Becton Dickinson and Co., Paramus, N.J.), and 0.1% (wt/vol) either lactose or glucose. The plates were incubated for 2 to 4 days at 30°C under a  $N_2$ - $CO_2$  (4:1) atmosphere or aerobically. The viable count was calculated by using six to eight plates, usually containing 50 to 300 CFU each. At zero time of starvation, a chemostat sample of *S. cremoris* Wg2 (dilution rate, 0.10  $h^{-1}$ ) with an  $A_{660}$  of 1 (0.2 mg of protein per ml) contained approximately  $10^9$  CFU/ml.

(ii) **Dilution method.** At the times during starvation indicated, *S. cremoris* Wg2 cells were diluted with MRS medium containing 1% (wt/vol) lactose to final CFUs of 0.1 to 100 per tube. The tubes were incubated at 30°C as described above. A dilution series consisted of 18 to 20 tubes in triplicate. The viable count was determined from the final tube in which growth could be detected.

Total counts were made in a Burkner chamber with a phase-contrast microscope.

**Measurement of  $Z\Delta pH$  and  $\Delta\psi$ .** The membrane potential ( $\Delta\psi$ ) and pH gradient ( $Z\Delta pH$ ) were determined from the distribution of [ $^3H$ ]tetraphenylphosphonium ion ( $TPP^+$ ) and [ $^{14}C$ ]benzoic acid, respectively, as described by Otto et al. (27) with the following modifications. [ $^3H$ ]  $TPP^+$  and [ $^{14}C$ ]benzoic acid were added to an actively growing culture of *S. cremoris* Wg2 at final concentrations of 27 nM and 0.75  $\mu$ M, respectively. Samples of 5 ml were collected, of which 4.0 ml was rapidly filtered over cellulose acetate filters (pore size, 0.45  $\mu$ m; Schleicher and Schüll, Dassel, Federal Republic of Germany). The binding of [ $^3H$ ]  $TPP^+$  to cell con-

stituents was estimated after deenergizing the cells by treatment with 2.5  $\mu$ M nigericin and 5.0  $\mu$ M valinomycin for 30 min at 30°C.  $\Delta\psi$  values were not corrected for concentration-dependent binding of  $TPP^+$ .

For washed-cell suspensions in phosphate-lactate buffer, [ $^3H$ ]  $TPP^+$  and [ $^{14}C$ ]benzoic acid were added to 27 nM and 1.33  $\mu$ M, respectively, 5 min prior to the addition of the energy source.

**Measurements of glycolytic intermediates.** Extraction of glycolytic intermediates was performed essentially as described previously (38). Samples were withdrawn from the chemostat by piercing the syringe needle through the rubber seal. The syringe contained 0.3 ml of ice-cold 28% (wt/vol) perchloric acid and 18 mM EDTA. Samples of 1.5 ml were collected. Further treatments were as described for the direct-extraction procedure by Otto et al. (26). To increase the metabolite concentrations in the cell extracts, the chemostat cultures were run at a limiting lactose concentration of 2.5%. Under these conditions, the steady-state protein concentration was about 1.2 mg/ml.

Glycolytic intermediates in cell extracts were assayed by fluorescence spectrophotometry with NADH- or NADP-coupled indicator systems. The enzymatic reactions utilizing the respective metabolites were as described by Maitra and Estabrook (19) with minor modifications. The reaction mixture (2.0 ml) contained 50 mM triethanolamine-KOH (pH 7.4), 50 mM KCl, and 5 mM  $MgCl_2$  buffer and included the appropriate concentrations of enzymes and other components. All reactions were conducted at room temperature, with 50 to 200  $\mu$ l of neutralized cell extract per assay. Intracellular metabolite concentrations were calculated from the difference between total and extracellular (i.e., supernatant fluid) concentrations.

**Measurement of amino acid concentrations.** Organisms were separated from the external medium by silicon oil centrifugation (35). Samples (1.1 ml) from the chemostat were rapidly transferred to microfuge tubes containing 0.8 ml of silicon oil (density, 1.03 g/ml) on top of 0.25 ml of 5% (wt/vol) perchloric acid and 9 mM EDTA. The mixture was centrifuged for 6 min at  $12,000 \times g$ . Fractions of 0.5 ml were taken from the supernatant, and, after removal of the remaining water and oil layers, the pellet was suspended in the perchloric acid layer. The extract was kept on ice for 20 min and subsequently centrifuged for 1 min. Finally, 0.175 ml of the extract was neutralized with an equal amount of 1 N KOH to  $KHCO_3$  solution. The samples were stored at -20°C. Supernatant fractions were used either to measure the amino acid concentrations in the growth medium or to measure the release of amino acids from the interior, i.e., when the cells were starved in phosphate-lactate buffer.

Amino acids were analyzed after derivation with dansyl chloride by using methods essentially described by Tapuhi et al. (33) and Wiedmeier et al. (46). Prior to the incubation with dansyl chloride, the amino acid samples were adjusted to pH 9.5 with saturated  $Na_2CO_3$ . Dansyl chloride (dissolved in acetonitrile) was added in 5- to 50-fold excess of the estimated total amount of amino acids per sample. The reaction proceeded at 37°C for 2 h and was stopped by the addition of 4.0 mM methylamine. The dansylated amino acids were separated by reversed-phase high-performance liquid chromatography on a C18 column ( $\mu$ Bondapak C18, 3.9 mm by 30 cm; Waters Associates, Inc., Milford, Mass.). The dansylated amino acids were eluted with a linear 0 to 55% (vol/vol) acetonitrile (in 30 mM sodium phosphate [pH 6.5]) gradient in about 35 min. Peak areas were calibrated with standard amino acid solutions. Intracellular amino acid

concentrations were calculated from the amount present in the cell extracts after correction for medium adhering to the cellular surface during silicon oil centrifugation (see below).

**Determination of the maximal rate of glycolysis.** Cell suspensions were washed twice and finally suspended to a density of 0.3 mg of protein per ml in 0.5 mM potassium phosphate (pH 6.4) containing 70 mM KCl and 1 mM  $\text{MgSO}_4$ . Following equilibration, lactose or glucose was added to a final concentration of 0.1% (wt/vol). pH changes were converted into nanomoles of  $\text{H}^+$  by calibration of the cell suspension with 1- $\mu\text{l}$  portions of 50 mM standardized HCl. The calibration curve was fitted with a polynomial function (of the third power) by using a program run on an Apple II minicomputer.

**Intracellular volume.** The specific intracellular volume was determined from the distribution of [ $^3\text{H}$ ]water and [ $^{14}\text{C}$ ]taurine by the procedure described by Bakker et al. (2). From the data, a specific internal volume of  $3.61 \pm 0.12 \mu\text{l}/\text{mg}$  and a ratio of extracellular water (adhering water after silicon oil centrifugation) over intracellular water of  $2.27 \pm 0.17$  could be calculated.

**Miscellaneous.** Protein was measured by the method of Lowry et al. (17) with bovine serum albumin as standard. ATP concentrations were determined by the firefly luciferase assay described by Lundin and Thore (18), and modified by Otto et al. (26). The lactate concentration in the culture fluid was determined by the method of Holdeman et al. (10).

**Materials.**  $^3\text{H}_2\text{O}$  ( $3.7 \times 10^7 \text{ Bq}/\text{ml}$ ), [ $\text{carboxyl-}^{14}\text{C}$ ]benzoic acid ( $207.2 \times 10^{10} \text{ Bq}/\text{mol}$ ), [ $\text{U-}^{14}\text{C}$ ]taurine ( $410.1 \times 10^{10} \text{ Bq}/\text{mol}$ ) and [ $^{14}\text{C}$ ]thiomethyl- $\beta$ -D-galactopyranoside ([ $^{14}\text{C}$ ]TMG) ( $3.7 \times 10^{10} \text{ Bq}/\text{mol}$ ) were obtained from the Radiochemical Centre, Amersham, England. [ $^3\text{H}$ ]tetraphenylphosphonium bromide ( $8,250 \times 10^{10} \text{ Bq}/\text{mol}$ ) was obtained from the Nuclear Research Centre, Negev, Beer Sheva, Israel. The silicon oil was made by mixing oils AR20 (density, 1.01 g/ml) and AR200 (density, 1.04) obtained from Wacker Chemicals, Munich, Federal Republic of Germany. Acetonitrile (spectrophotometric grade) was obtained from Janssen Chimica, Beerse, Belgium. All other chemicals were reagent grade and were obtained from commercial sources.

## RESULTS

**Viability.** In continuous cultures, starvation for the growth-limiting substrate was initiated by switching off the medium supply without causing additional stress to the organisms. The viable count of the culture was followed by diluting cell samples, taken aseptically at various time intervals, either with buffer or with the effluent of the chemostat after removal of the cells. The composition of the dilution media had little influence on the viability of *S. cremoris* Wg2 as long as the buffers contained potassium (50 mM) and magnesium (1 mM) ions. The viable count as determined by the plate count method or by the dilution method was the same. Incubation of the plates aerobically or under a  $\text{N}_2\text{-CO}_2$  (4:1) atmosphere yielded similar viable-count numbers.

The effect of starvation on the viability of *S. cremoris* Wg2 cells previously grown in continuous culture at various dilution rates was studied (Fig. 1). The percentage of CFU in a chemostat-grown culture of *S. cremoris* was usually 70 to 80% of the CFU observed microscopically, independent of the dilution rate. The physiological state, i.e., the imposed growth rate, of *S. cremoris* cells upon entering the starvation phase strongly affected their ability to survive periods of energy starvation. The death rates, i.e., the reciprocal of the time in which 50% of the culture lost viability, were 0.029,

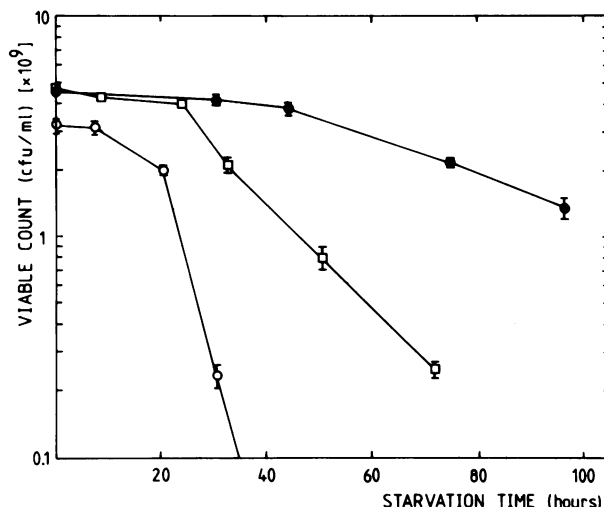


FIG. 1. Effect of the imposed growth rate on the viable count of *S. cremoris* Wg2 cells during lactose starvation. Lactose-limited cells of *S. cremoris* were grown at a dilution rate of  $0.07 \text{ h}^{-1}$  (●),  $0.11 \text{ h}^{-1}$  (□), and  $0.38 \text{ h}^{-1}$  (○) prior to starvation in the culture fluid. The lactose concentration in the medium reservoir was 2.5% (wt/vol). Cell samples were diluted with sterile effluent from the chemostat on the basis of total count measurements. The CFUs were calculated by using six to eight agar plates.

0.076, and  $0.298 \text{ h}^{-1}$  for cultures grown at 0.07, 0.11, and  $0.38 \text{ h}^{-1}$ , respectively. At all dilution rates, the viable count decreased logarithmically over time.

*S. cremoris* Wg2 usually exists in chains of cells with different lengths. Consequently, the number of viable cells determined from the number of CFU multiplied by the average chain length could be overestimated. The error will increase with increasing cell number per chain. In the experiment described above, the average chain length increased from 2.5 to 3.8 cells when the dilution rate was raised from 0.07 or 0.11 h to  $0.38 \text{ h}^{-1}$ . This was also reflected in the lower viable count number with cells grown at a rate of  $0.38 \text{ h}^{-1}$  at the beginning of starvation (time zero, Fig. 1). The average chain length of *S. cremoris* Wg2 cells decreased somewhat in the first hours of starvation. Interestingly, individual cocci were hardly ever observed, even after prolonged storage in the starvation media (data not shown).

**Energy metabolism during the initial stage of lactose starvation.** During steady-state growth in a lactose-limited chemostat, the intracellular concentrations of glycolytic intermediates of *S. cremoris* Wg2 are very dependent on the dilution rate. The sugar-phosphate pool, i.e., fructose 1,6-diphosphate, fructose 6-phosphate, glucose 6-phosphate, dihydroxyacetone phosphate, and glyceraldehyde 3-phosphate, increased, whereas the PEP pool, i.e., 3-phosphoglycerate, 2-phosphoglycerate, and PEP, decreased with increasing dilution rate (data not shown). The concentrations of these intermediates at a dilution rate of  $0.1 \text{ h}^{-1}$  are given in Fig. 2. When the starved state was entered, opposite initial changes occurred in the sugar-phosphate and PEP pools. The sugar-phosphate pool, which consisted mainly of fructose 1,6-diphosphate, depleted within 5 min after the interruption of the medium supply. The PEP pool, on the other hand, increased during the first 5 min of lactose starvation, after which these intermediates also disappeared. The depletion of the PEP pool between 10 and 30 min of lactose starvation indicates that the activity of pyruvate kinase is low but still significant, i.e., approximately 1.2

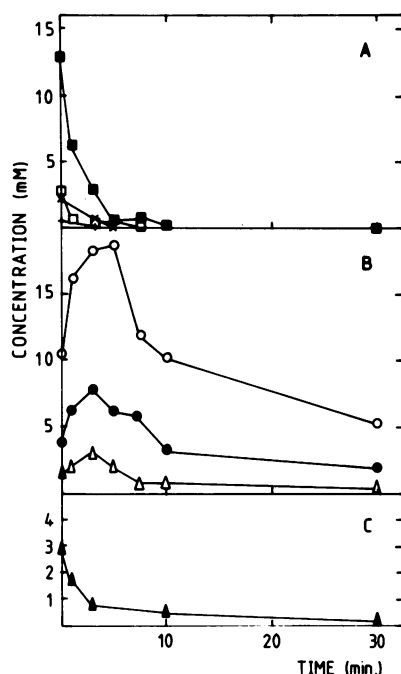


FIG. 2. Time course of concentration changes of glycolytic intermediates in cells of *S. cremoris* Wg2 during lactose starvation. The chemostat was run at a dilution rate of  $0.10 \text{ h}^{-1}$  with 2.5% (wt/vol) lactose as the growth-limiting substrate. At zero time of starvation, the medium supply was turned off. (A) The sugar phosphates: glucose 6-phosphate ( $\times$ ), fructose 6-phosphate (+), fructose 1,6-diphosphate ( $\blacksquare$ ), and dihydroxyacetone phosphate plus glyceraldehyde 3-phosphate ( $\square$ ). (B) The PEP-pool intermediates: 3-phosphoglycerate ( $\circ$ ), PEP ( $\bullet$ ), and 2-phosphoglycerate ( $\Delta$ ). (C) ATP ( $\blacktriangle$ ).

nmol of PEP per mg of protein per min, despite the absence of glycolytic activators (sugar-phosphates) and the presence of 100 to 240 mM phosphate (28). Thompson and Thomas (43) approximated the rate of PEP utilization by starved *S. lactis* ML3 at  $0.17 \text{ nmol/mg}$  (dry weight) of cells per min. The internal concentration of ATP was about 3 mM in growing cells. Also, this ATP pool decreased rapidly upon starvation. After 1 h of starvation, the concentrations of all three groups of phosphorylated intermediates were below 0.1 mM.

The accumulation of the nonmetabolizable lactose analog TMG has been shown to correlate quantitatively well with the sum of the intracellular concentrations of 3-PG, 2-PG, and PEP (40, 43). The uptake of TMG by *S. cremoris* Wg2 cells, which were starved either in growth medium without lactose or in buffer as described by Thompson and Thomas (43) indicated that the latter conditions favor the maintenance of the PEP pool (data not shown). Preliminary experiments, in which redox mediators were used to modulate the activity of pyruvate kinase in vitro, suggested that the differences in maintaining the PEP pool could be caused by the differences in the redox state of the environments.

The other important energy intermediate is the proton motive force. The magnitudes of the components of the proton motive force were monitored during starvation. Both  $Z\Delta\text{pH}$  and  $\Delta\psi$  dropped to zero within 90 min after the medium supply had been interrupted (Fig. 3). The steady-state values of  $Z\Delta\text{pH}$  and  $-\Delta\psi$  were 32 and 102 mV, respectively.

**Release of amino acids from starved *S. cremoris* cells.** The intra- and extracellular concentrations of amino acids of *S.*

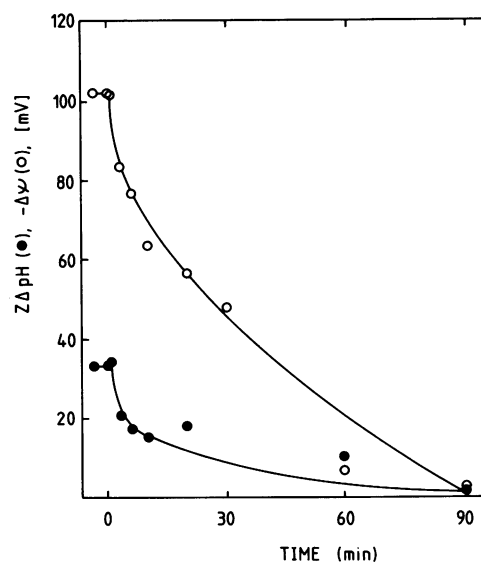


FIG. 3. Decrease of the proton motive force upon the onset of lactose starvation (zero time) in *S. cremoris* Wg2 cells. *S. cremoris* was grown in a lactose (0.5% [wt/vol])-limited chemostat at a dilution rate of  $0.10 \text{ h}^{-1}$ . A steady state was established before the medium supply was turned off. The membrane potential ( $\Delta\psi$ ) ( $\circ$ ) and the transmembrane proton gradient ( $Z\Delta\text{pH}$ ) ( $\bullet$ ) were determined as described in Materials and Methods.

*cremoris* Wg2 growing in continuous culture at a dilution rate of  $0.10 \text{ h}^{-1}$  are given in Table 1. The intracellular amino acid pools accounted for 1.6% of the cell dry weight. Glutamate accounted for almost 50% of the total free amino acid pool. Glutamine and asparagine, which were not present in the growth medium, were found intracellularly in small quantities. These amino acids may be synthesized from glutamate and aspartate by glutamine and asparagine synthetase, respectively. Although most amino acids were accumulated against a large concentration gradient, thermodynamic equilibrium between the proton motive force and

TABLE 1. Intracellular amino acid pools and amino acid concentration gradients in growing *S. cremoris* Wg2 cells<sup>a</sup>

Amino acid	Intracellular concn (mM)	Extracellular concn (mM)	Concn gradient
Leu <sup>b</sup> + Ile <sup>b</sup>	14.5	1.44	10.1
Val <sup>b</sup>	14.5	1.75	8.3
Ala	13.3	0.94	13.9
Gly	14.4	0.95	15.1
Glu <sup>b</sup>	107.6	2.37	45.4
Asp	13.8	0.39	35.4
Gln	2.0	0	
Asn	0.5	0	
Lys <sup>b</sup>	16.4	2.28	7.2
His <sup>b</sup>	2.2	0.88	2.5
Arg <sup>b</sup>	5.9	1.34	4.4
Trp + Phe <sup>b</sup>	2.2	0.61	3.6
Tyr <sup>b</sup>	4.1	1.17	3.5
Thr	6.1	0.60	10.1
Ser	0.4	0.36	1.1
Pro <sup>b</sup>	11.2	4.48	2.5
Met <sup>b</sup>	1.2	0.86	1.4

<sup>a</sup> *S. cremoris* cells were grown in a lactose (0.5% [wt/vol])-limited chemostat at a dilution rate of  $0.10 \text{ h}^{-1}$ . Amino acid concentrations were measured in steady-state cultures, as described in Materials and Methods.

<sup>b</sup> Amino acids essential for the growth of *S. cremoris* Wg2 (R. Otto, unpublished data).

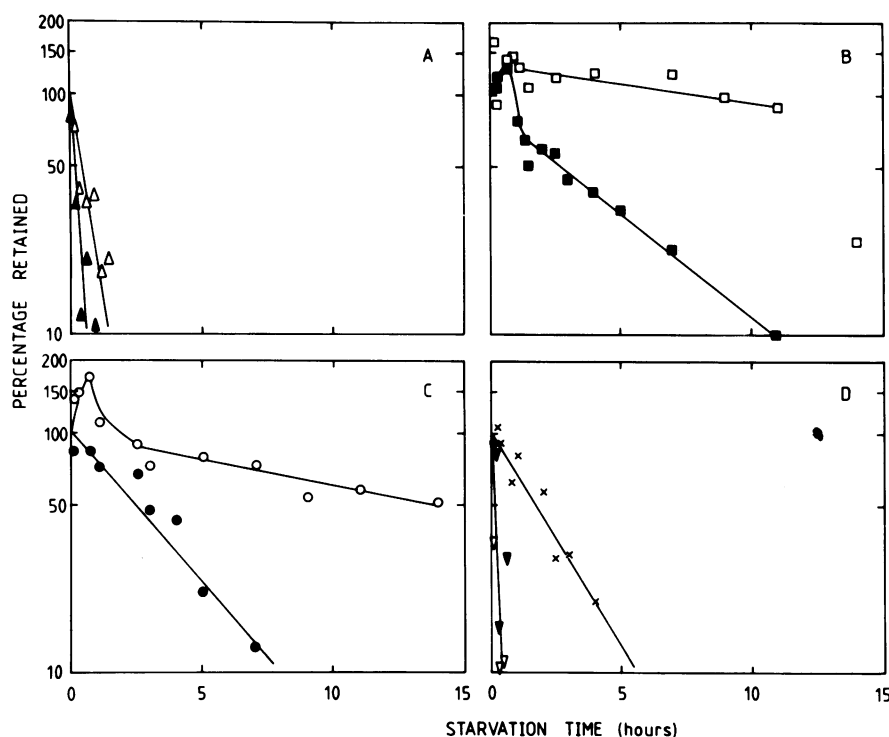


FIG. 4. Release of amino acids from *S. cremoris* Wg2 cells during lactose starvation. Cells were grown at a dilution rate of  $0.10\text{ h}^{-1}$  and with 0.5% (wt/vol) lactose as the growth-limiting substrate. At zero time of starvation, the medium supply was turned off. The decrease in the intracellular amino acid pools with starvation time are shown. (A) Leu plus Ile ( $\Delta$ ) and Val ( $\blacktriangle$ ); (B) Ala ( $\square$ ) and Gly ( $\blacksquare$ ); (C) Glu ( $\circ$ ) and Asp ( $\bullet$ ); (D) Thr ( $\nabla$ ), His ( $\blacktriangledown$ ), and Trp plus Phe ( $\times$ ).

the amino acid gradients was never achieved (assuming that  $1\text{ H}^+$  accompanies the uptake of 1 amino acid). If the proton motive force or one of its components is the driving force for amino acid transport (7; B. Poolman, E. J. Smid, and W. N. Konings, manuscript in preparation), a rapid release of these solutes from the cell upon lactose starvation can be expected. Several amino acids, i.e., Leu, Ile, and Val (Fig. 4A); Thr and His (Fig. 4D); and Met, Pro, Lys, and Tyr (data not shown) indeed leaked out from the cell at rates that

correlated with the dissipation of the proton motive force (Fig. 3). The half-times of efflux,  $t_{(0.5)}$ , for Gly, Ala, Asp, Glu, and Trp plus Phe, on the contrary, were 215, 600, 165, 840, and 110 min, respectively (Fig. 4B to D). With the exception of Glu, all amino acids eventually equilibrated with the concentrations in the external medium (Table 1). At the onset of starvation, an increase of the intracellular concentration was observed for the amino acids that were retained longest by the cell i.e., Gly, Ala, and Glu.

To quantitate the release of amino acids into the external medium, *S. cremoris* cells were washed free of residual growth medium and suspended in a phosphate-lactate buffer containing potassium phosphate, sodium lactate, and  $\text{MgSO}_4$ . The sum of the amino acids released after washing of the cells at up to 47 h of starvation could account for more than 90% by the release of amino acids from the free intracellular pool (data not shown), indicating that very little protein breakdown had occurred.

**Response of starved *S. cremoris* cells upon readdition of lactose.** The generation of the proton motive force can be considered to be the result of the energy metabolism of *S. cremoris*. For this, the activity of the Embden-Meyerhof pathway together with a functional sugar uptake system and the  $\text{F}_0\text{F}_1$ -ATPase are required. Figure 5 shows the time course for the generation of the  $\Delta\psi$  and the  $\text{Z}\Delta\text{pH}$  after different periods of lactose starvation in a phosphate-lactate buffer at pH 6.4. The proton motive force had already fallen to zero at the first moment of lactose feeding. After the first lactose pulse, the absolute values of  $\Delta\psi$  and  $\text{Z}\Delta\text{pH}$  in the steady state were approximately 30 and 20 mV, respectively, higher than for cells growing in a lactose-limited chemostat. This increase of the proton motive force is partly due to the difference in composition between the phosphate-lactate buffer and the growth medium (data not shown). The rate of

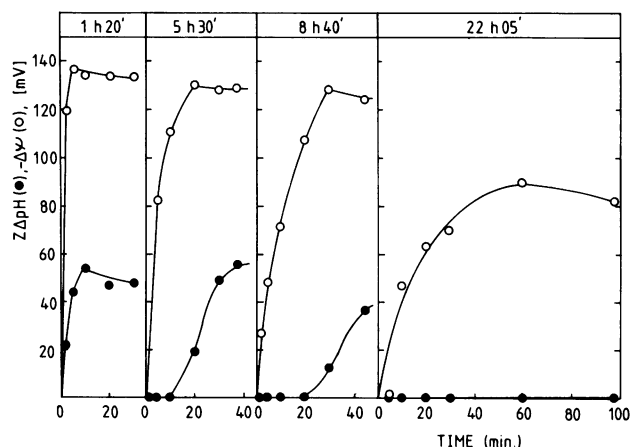


FIG. 5. Time course for the generation of  $\Delta\psi$  and  $\text{Z}\Delta\text{pH}$  by *S. cremoris* Wg2 cells after various periods of lactose starvation. Cells, taken from a chemostat run at a dilution rate of  $0.10\text{ h}^{-1}$ , were starved in phosphate-lactate buffer at a final protein concentration of 0.25 mg/ml.  $[^3\text{H}]\text{TPP}^+$  and  $[^{14}\text{C}]\text{benzoic acid}$  were added 5 min prior to the addition of 0.1% lactose (zero time).

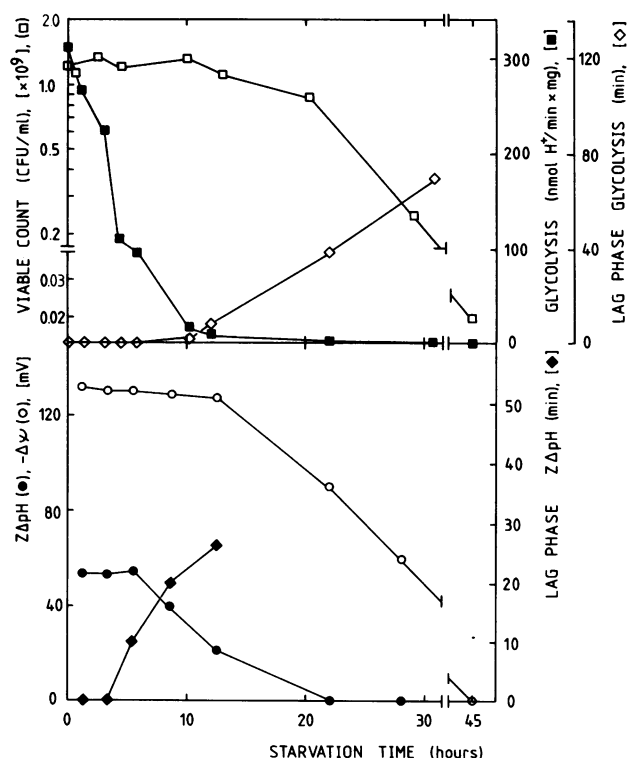


FIG. 6. Effect of starvation on the glycolytic activity, the regeneration of the proton motive force, and the viability of *S. cremoris* Wg2 cells. Cells were grown and starved as described in the legend to Fig. 5. The data for the steady-state values of  $\Delta\psi$  (○) and  $Z\Delta pH$  (●) and the lag phase in  $Z\Delta pH$  formation (◆) were obtained from  $\Delta\psi$  and  $Z\Delta pH$  progress curves (Fig. 5). The rates of glycolysis (■) were determined from the linear part of the graphs in which the acid production (nanomoles of  $H^+$ ) was plotted against time. The lag time of glycolysis (◇) was defined as the time between the addition of lactose and the time when glycolysis proceeded linearly, i.e., maximally. Culture viability (□) was determined by the plate count method, as described in Materials and Methods.

$\Delta\psi$  and  $Z\Delta pH$  generation decreased with increasing starvation time, which ultimately leads to decreased steady-state values of the proton motive force (Fig. 5). These results are compared with the results of viable-count measurements and of the glycolytic activity (Fig. 6). In the initial phase of starvation, the steady-state values of the proton motive force after refeeding with lactose remained essentially constant, despite the drastic changes that were observed in the maximal glycolytic activity. Within 10 h of lactose starvation, the maximal rate of glycolysis had fallen to less than 10% of the activity in the growing culture. The decreased activity of glycolysis is expressed as a diminished rate of ATP synthesis (28) and consequently as a decreased ability to regenerate a proton motive force. The capacity to generate a  $\Delta\psi$  and  $Z\Delta pH$  is given as the time required to reach steady-state values. It appeared that upon starvation the capacity to generate a  $\Delta\psi$  and  $Z\Delta pH$  decreased linearly with the maximal rate of glycolysis (Fig. 7). The steady-state values of the proton motive force were not affected as long as the rate of glycolysis remained above 50 nmol of  $H^+$  per mg of protein per min.

The fall in the maximal rates of glycolysis with increasing starvation times was similar with glucose and lactose as fermentable substrates. Also, the survival of *S. cremoris*

Wg2 cells on agar plates with either lactose or glucose as substrates was the same (data not shown). Interestingly, the lag phase in glycolysis, i.e., the period before acid production proceeded linearly with time, was not observed before 30 h of starvation when glucose was used as substrate (data not shown), whereas a gradually increasing lag phase was observed after 10 h of lactose starvation (Fig. 6).

**Effect of starvation on amino acid transport.** The uptake of amino acids in starved cultures of *S. cremoris* Wg2 was measured after the addition of lactose. The cells had previously been grown in a lactose-limited chemostat at a dilution rate of  $0.11\ h^{-1}$  and were starved in the culture fluid by having the medium supply switched off. The ability to accumulate most amino acids diminished with starvation time in a similar manner as the decrease in the regenerated proton motive force (data not shown). In contrast to these amino acid carriers, which respond to a decrease in the proton motive force, the transport systems for Asp and Glu appeared to be very sensitive to lactose starvation. For example, *S. cremoris* Wg2 had already lost more than 50% of its potential to accumulate Asp and Glu when culture viability was still 100%.

## DISCUSSION

Continuously cultured cells of *S. cremoris* Wg2 grown under lactose limitation appeared to survive longer when the imposed growth rate before the cells entered the starvation phase was lower (Fig. 1). The percentage of viable cells in the steady state of growth was independent of the dilution rate. It is therefore unlikely that survival at different dilution rates was influenced by the presence of dead cell materials that supplied substrates for cryptic growth. Furthermore, the survival of *S. cremoris* cells kept in growth medium or in buffer was very similar. An apparent increase in the viable count which sometimes was observed in the first hours of starvation could be ascribed to an increased total count and a decreased average chain length. Increased survival after growth at lower dilution rates has been explained by a better

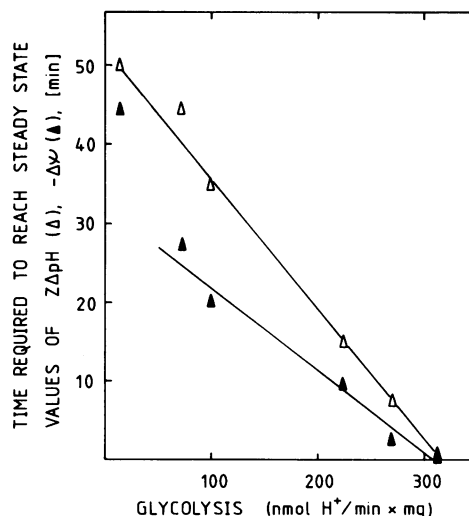


FIG. 7. Relationship between the capacity to generate a proton motive force and the glycolytic activity. Cells were grown and starved as described in the legend to Fig. 5. The capacity to generate  $Z\Delta pH$  (Δ) and  $\Delta\psi$  (▲), expressed as the time required to reach steady-state values, was determined from  $Z\Delta pH$  and  $\Delta\psi$  progress curves (Fig. 5). The glycolytic activity was determined after various periods of starvation, as described in the legend to Fig. 6.



adaptation to starvation through lower growth rates (9, 22, 23), but the opposite results have also been obtained (29). An effect of the growth phase on the survival time during starvation for a batch-grown genetically uniform population of *S. lactis* ML3 has not been observed (36). It is unlikely that our viable count measurements were influenced by genetic changes during cultivation in the chemostat, since the results were obtained independent of the order of the imposed growth rates.

There have been conflicting reports on the role of  $P_i$  in the regulation of pyruvate kinase (20, 28, 44). The increase in the PEP pool in streptococci when the starvation phase was entered has been attributed to the inhibition of pyruvate kinase by  $P_i$  (20, 44). The maintenance of a large PEP pool under conditions of energy starvation would supply the cell with an energy reserve that could increase the response of a starved culture upon renewed addition of an exogenous energy source (5, 43). Strikingly, the period for which *S. lactis* ML3 remained viable correlated well with the time the cell was furnished with the PEP pool. Our experiments indicate that concentrations of the PEP pool intermediates in continuously cultured *S. cremoris* cells were already below 0.1 mM after 1 h of lactose starvation while the cells were still fully viable.

The transient accumulation of PEP pool intermediates (Fig. 2B) takes place over a period during which the phosphate pool increases only slightly (28). The concentration of pyruvate kinase activators, on the other hand, decreases more than 20-fold during this starvation time (Fig. 2A). In fact, the sugar-phosphate pool increases concomitantly with the drop in the phosphate pool in the experiments in which Thompson and Torchia (44) attempted to demonstrate that pyruvate kinase is reactivated in vivo by a decrease in free inorganic phosphate (Fig. 8 in reference 44). The activity of pyruvate kinase of *S. cremoris* is affected by  $P_i$  in a similar manner to that of *S. lactis* (20). However, the transient increase in the PEP pool of *S. cremoris* at the onset of starvation is more likely to be explained by a decrease in the sugar-phosphate pool and the absence of PEP consumption by the phosphotransferase transport system than by an increase in free inorganic phosphate.

The glycolytic metabolites have been shown to disappear rapidly from chemostat-grown cells of *S. cremoris* upon lactose starvation. From the titration of the acid production in continuously cultured (lactose-limited) *S. cremoris* cells at a dilution rate of  $0.1\text{ h}^{-1}$ , a steady-state glycolytic activity of about 300 nmol of  $H^+$  per min per mg of protein can be calculated. This indicates that pyruvate kinase is highly active, despite the presence of 70 mM  $P_i$  (28). The conflicting results with respect to the maintenance of a PEP pool in starved streptococci (references 20 and 44 versus 28 and this study) suggest that in addition to activation by sugar-phosphates and inhibition by phosphate, the activity of pyruvate kinase is modulated by other factors.

It has frequently been observed that the size of the free amino acid pool in gram-positive organisms is 10-fold higher than that in gram-negative organisms (8, 34). Although the size and composition of the amino acid pool may vary considerably with the dilution rate, pH, growth-limiting substrate, and other environmental factors (8), the concentrations given in Table 1 resemble those for other streptococci (8, 37). The sum of the concentrations of amino acids in the free intracellular pool in *S. cremoris* is 230 mM under the conditions described, to which glutamate contributes almost 50%.

To monitor the ultimate bioenergetic response of starved

*S. cremoris* cells in relation to their viability, the regeneration of the components of the proton motive force was measured (Fig. 5 and 6). The rate of proton motive force generation decreased from the initial hours of starvation without having an effect on the steady-state values in the first 12 h. Owing to the relatively low electrical capacitance of the cytoplasmic membrane in comparison with the differential buffering capacitance of the system (24), a decreased primary pump activity has more pronounced effects on the formation of the  $\Delta pH$  than on the  $\Delta\psi$ . The decreased ability to generate a proton motive force is caused by a decreased glycolytic activity (Fig. 6), leading to a diminished ATP production necessary for proton extrusion. The effect of starvation on the maximal rate of glycolysis corresponds well to the effects on the maximal rate of ATP synthesis (28). Since generalized, nonspecific protein degradation is insignificant in the initial phase of starvation, the question arises of what causes the rapid fall in the glycolytic activity. The lag phase preceding maximal glycolytic activity differed when either lactose or glucose was used as substrate, but the actual rate of glycolysis did not. This indicates that the sugar translocation step itself is not directly affected by starvation. Lactose and glucose are taken up by separate transport systems which may share, however, a number of cytoplasmic components (21, 41, 42). It is unlikely that leakage of (adenine) nucleotides affected the glycolytic activity, since *S. cremoris* cells are able to synthesize high concentrations of ATP even after 24 h of starvation (28). We are currently investigating whether particular enzymes, including the PEP-dependent sugar transport systems, are exposed to specific proteolysis or whether other mechanisms cause the sensitivity of glycolysis toward lactose starvation.

A role for specific protein degradation in the survival of starved bacteria may be found in the breakdown or inactivation of enzymes involved in futile cycles or in the protection of an organism against substrate-accelerated death (3). In fact, it has frequently been observed that catabolic enzymes are the target of inactivation during starvation (32). From the amino acids appearing in the external medium, it can be calculated that general nonspecific protein degradation is insignificant during the first 40 h of starvation. Furthermore, there is no evidence that amino acids (with the exception of arginine catabolism in *S. lactis*) can be used as endogenous substrates by group N streptococci (37).

To respond immediately to a sudden appearance of nutrient, it is essential for an organism to keep transport systems functional for essential nutrients during starvation periods (5). Since a large number of amino acids are essential for the growth of *S. cremoris* (Table 1), it is likely that the capacity to replenish the amino acid pools is closely related to survival. The effect of starvation on the activity of the proton motive force-dependent transport systems appears merely to be determined by the magnitude of the proton motive force, indicating that intrinsic losses of transport activity do not occur. The central role of the proton motive force during energy starvation of *S. cremoris* Wg2 is further strengthened by the apparent correlation between the viable-count number and the capacity to generate a proton motive force (Fig. 6). This conclusion is at variance with the conclusion reached for *Staphylococcus epidermidis* (11). Although it is not possible to discriminate between a proton motive force generated by viable cells only or one generated by both viable and nonviable cells, the data can be explained quantitatively by the former possibility.

The ability of *streptococcus* species to survive periods of energy starvation may be poor when compared with marine



bacteria (1, 25). However, depending on the habitat of an organism, advantages may not only be found in the ability to survive extended periods of starvation but may for instance also be related to a quickly responding metabolism in a rapidly changing environment.

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